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14. ABSTRACT
We characterized one mechanism how BRCA1, a downstream effector of MDC1 and Chk2, in maintaining genomic stability. We show that BRCA1 interacts with topoisomerase II and regulates topoisomerase II activity and localization. In addition, we characterized the interaction between MDC1 and DNA-PKcs/Ku, an upstream regulator of Chk2. We show that MDC1 regulates DNA-PKcs phosphorylation and localization, and participates in DNA repair. Finally, we generated MDC1-/- mice and in the process of evaluating the physiological function of MDC1. Our preliminary data suggest that MDC1 plays an important role in DNA damage response and maintaining genomic stability. In summery, our studies revealed novel mechanisms of the DNA damage response pathway and our animal model of the MDC1 knockout mice will elucidate how dysfunction of the Chk2-MDC1 pathway contributes to the development of tumor.

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DNA damage, DNA repair, cell cycle, knockout, protein-protein interaction, signal transduction, tumorgenesis, tumor suppressor

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Introduction

We propose to investigate the functional interaction between Chk2 and Kiaa0170. Chk2 is a critical regulator of DNA damage response pathway and a tumor suppressor. Mutations in Chk2 confer an increased risk of breast cancer (Bell et al., 1999; Meijers-Heijboer et al., 2002; Vahteristo et al., 2002). However, the regulation of the Chk2-mediated pathway is still not clear. Our preliminary studies show that Kiaa0170 (renamed as MDC1, Mediator of DNA Damage Checkpoint Protein 1), a newly identified nuclear protein, associates with Chk2 after ionizing radiation (IR). We proposed to study the molecular mechanism of the MDC1-Chk2 interaction and the functional role of MDC1 in the Chk2-dependent DNA damage response pathway. These studies will provide new insights into the regulation of the DNA damage response pathway and the development of tumor. They might also reveal new strategies for tumor therapy and prevention.

Body

The specific aims 1is to define and characterize the interacting domains between Chk2 and MDC1. Our *in vitro* and in *vivo* studies during the first year of fellowship (Aug. 03-Aug. 04) defined the molecular mechanism of MDC1-Chk2 interaction (Lou et al., 2003b). Therefore we mostly completed Specific Aim 1 described in Statement of Work.

During the first year, we also investigated the functional role of MDC1 in Chk2-dependent DNA damage-signaling pathway, as proposed in Specific Aim 2. Using small interfering RNA (siRNA) to suppress MDC1 expression, we show that MDC1 regulates intra-S checkpoint, p53 phosphorylation as well as radiation-induced apoptosis, both of which are regulated by Chk2. In addition, we show that the interaction between Chk2 and MDC1 is important for these Chk2-dependent functions (Lou et al., 2003b). Furthermore, we found that MDC1 regulates BRCA1, another critical suppressor of breast cancer (Scully and Livingston, 2000) and a downstream signaling molecular of Chk2. MDC1 interacts with BRCA1 constitutively, and regulates BRCA1 foci formation and phosphoryaltion following DNA damage (Lou et al., 2003a). These studies established MDC1 as an important mediator of the DNA damage response pathway.

During the second year of the fellowship (Aug. 2004-Aug.2005), we continued to investigate the signaling pathways regulated by MDC1 and Chk2. BRCA1 is a downstream signaling molecular of MDC1 and Chk2 that plays an important role in maintaining genomic stability. However, how BRCA1 maintains genomic stability is not clear. We previously observed that BRCA1 not only forms nuclear foci in response to DNA damage, it also forms foci during DNA replication (S-phase). We set out to investigate the functional significance of BRCA1 foci formation during S-phase. We found that BRCA1 interacts and colocalizes with topoisomerase II in S-phase foci. The major role of topoisomerase II is to separate DNA strands following DNA replication. We hypothesized that BRCA1 is involved in DNA decatenation. Our *in vitro* studies confirmed that BRCA1 regulates DNA decatenation. In addition, BRCA1-deficient cells

show defective chromosome segregation. We further show that BRCA regulates DNA decatenation by influencing topoisomerase II activity and localization. These studies suggest that BRCA1 maintains genomic stability by regulating DNA decatenation. These studies were published at Nature Structural and Molecualr Biology (12:589-563, Appendix 1)

During the same period, we made further structure-function analysis of MDC1. MDC1 has three functional domains: the N-terminal FHA domain, the C-terminal BRCT domain and a central repeat region. The repeat region is composed of 14 repeats of 41 amino acids. We and others have shown that the FHA domain of MDC1 interacts with Chk2 and MRE11 (Goldberg et al., 2003; Lou et al., 2003b). To investigate the binding partner of the repeat region of MDC1, we did affinity purification using GST-MDC1 repeats. We isolated DNA-PKcs/Ku as a binding partner of the MDC1 repeat region. We further show that MDC1 regulates DNA-PKcs phosphorylation and DNA-PK-dependent DNA repair function. These studies were published at J. Biol. Chem. (**279**:46359-62, Appendix 2).

All previously studies were performed using cancer cell lines *in vitro*. However, it is still not clear about the physiological function of MDC1. To understand the physiological function of MDC1 and its role in the DNA damage response pathway *in vivo*, we generated MDC1 knockout mice (Figure 1).

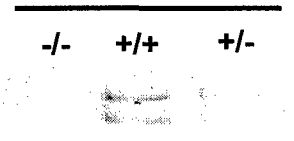

Figure 1. Cell extracts were prepared from testes of mice of indicated phenotypes blotted with antibodies against N-terminus of MDC1.

The MDC1$^{-/-}$ mice were growth retarded, similar to ATM$^{-/-}$ and H2AX$^{-/-}$ mice (Barlow et al., 1996; Celeste et al., 2002; Elson et al., 1996; Xu et al., 1996). The average weight of 5-month old MDC1$^{-/-}$ mice was 80% of that of wild-type mice (Figure 2). The defective growth of MDC1$^{-/-}$ mice correlated with the reduced proliferation of MDC1$^{-/-}$ cells *in vitro* (Figure 3). Mouse embryonic fibroblast (MEFs) from MDC1$^{-/-}$ mice grew poorly *in vitro* due to a decrease in actively dividing cells, judged by BrdU staining. This was accompanied by the accumulation of giant nondividing cells,

possibly due to premature senescence (data not shown). Since DNA damage is known to induce premature senescence, these observations agree with a role of MDC1 in DNA damage response.

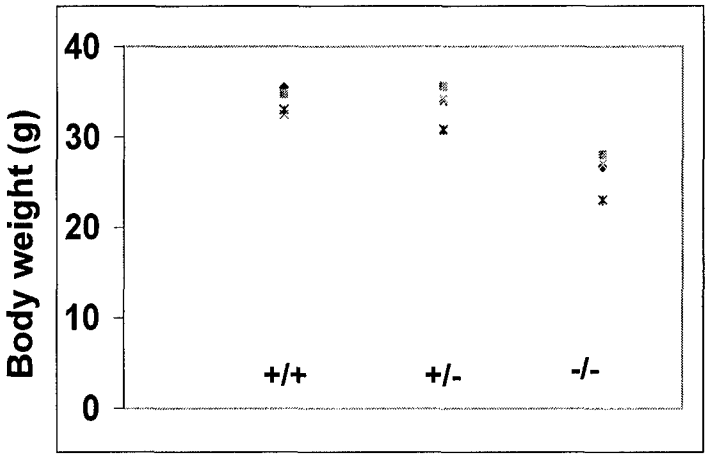

Figure 2. MDC1$^{-/-}$ mice show growth retardation and male infertility. Body weights of male MDC1$^{+/+}$, MDC1$^{+/-}$ and MDC1$^{-/-}$ mice were evaluated at 5 months of age. Y axis represents body weight (g).

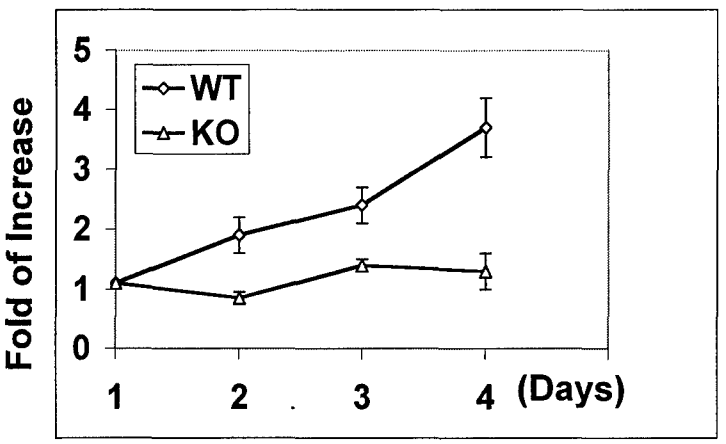

Figure 3. MDC1$^{-/-}$ MEFs showed reduced proliferation *in vitro*. MDC1$^{+/+}$and MDC1$^{-/-}$ MEFs were cultured in 6-well plates at a density of 10^5/well. Each day, one set of cells was trypsinized and counted. At day 3, cells were split and replated into larger plates. Y axis represents folds of increase in cell numbers.

One of the hallmarks of defective DNA damage responses is increased radiation sensitivity. To test if the loss of MDC1 expression renders mice hypersensitive to ionizing radiation (IR), we irradiated MDC1$^{+/+}$, MDC1$^{+/-}$ and MDC1$^{-/-}$ mice. As shown in Figure 4, all MDC1$^{-/-}$ mice died within 16 days after 7 Gy of irradiation, while 80% of MDC1$^{+/+}$and MDC1$^{+/-}$ mice were still alive two months after irradiation.

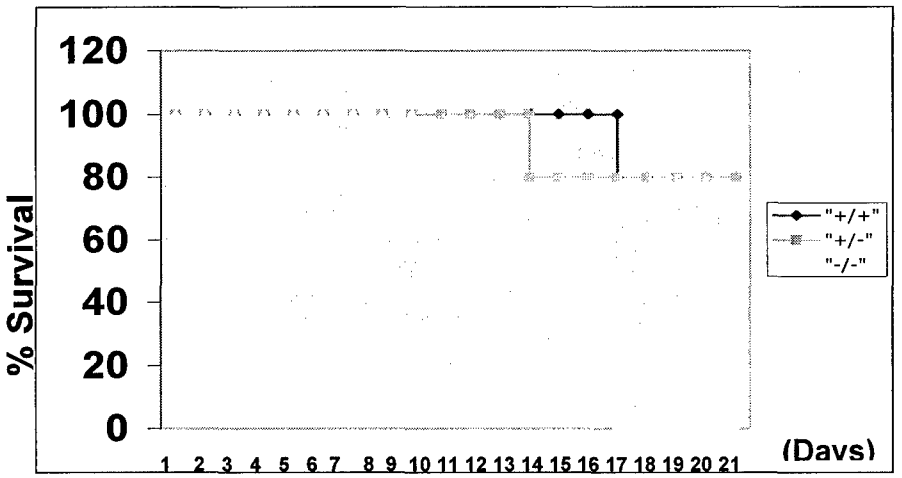

Figure 4. Survival curve of $MDC1^{+/+}$ and $MDC1^{-/-}$ littermates after whole body exposure to 7 Gy of IR.

On the cellular level, $MDC1^{-/-}$ MEFs are also hypersensitive to low-dose irradiation (Figure 5). These results support an important role of MDC1 in the DNA damage response pathway.

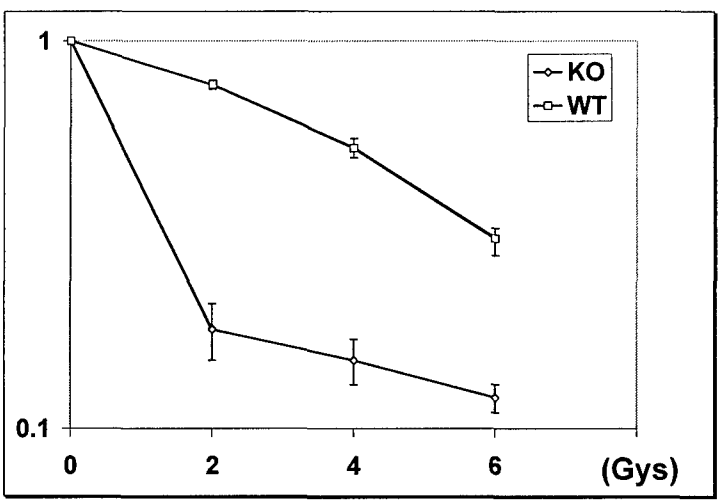

Figure 5. Increased radiation sensitivity was observed in passage 1 $MDC1^{-/-}$ MEFs. Y axis represents the percentage of surviving cells relative to un-irradiated control cells of the same genotypes.

To evaluate the physiological role of MDC1 in maintaining genomic stability, we used metaphase spreads of activated T cells to examine chromosome aberrations of $MDC1^{+/+}$ and $MDC1^{-/-}$ cells in the absence of exogenous DNA damaging agents. Few $MDC1^{+/+}$ cells (3.5%) displayed spontaneous chromosome aberrations (Figure 6,7). In contrast, significantly more $MDC1^{-/-}$ cells (17.5%) show spontaneous chromosome aberrations, including chromosome breaks, chromatid breaks, fragments, dicentric chromosomes (Figure 6,7).

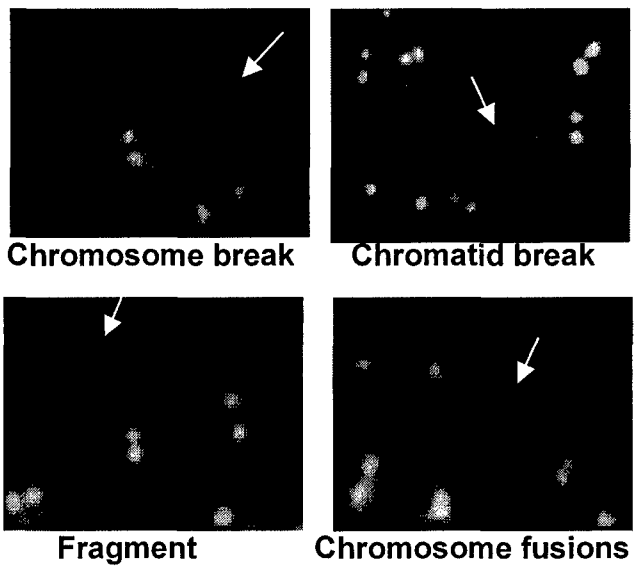

Figure 6. Examples of chromosomal aberrations in MDC1$^{-/-}$ splenocyte metaphases. Metaphase spreads were hybridized with a telomere Cy3-labeled PNA probe and counterstained with DAPI.

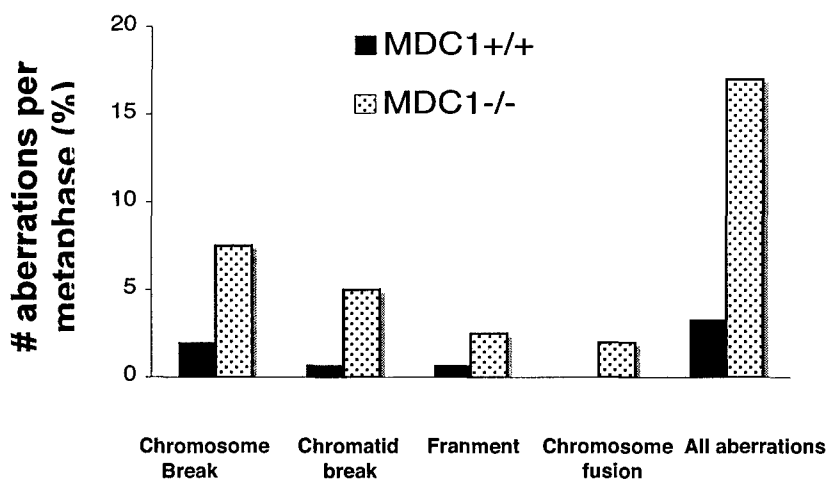

Figure 7. Numbers and spectrum of chromosomal aberrations in 7 MDC1$^{-/-}$ and 6 MDC1 control (5 MDC1$^{+/+}$ and 1 MDC1$^{+/-}$) cultures of CD43^{+} splenocytes incubated with concanavalin A (2.5 μg/mL) for 72 hr. Number of aberrations for each mouse is calculated as (number of aberrations/number of metaphases analyzed)*100. Columns represent average of 6 or 7 mice (control and knockout respectively).

These results suggest that MDC1 plays an important role in maintaining genomic stability *in vivo*.

We are currently studying the mechanism of how MDC1 functions in the DNA damage response pathway and maintains genomic stability. Specifically, we will use MEFs from MDC1$^{+/+}$ and MDC1$^{-/-}$ mice and examine Chk2 localization and activation. In addition, we will assess the role of MDC1 in Chk2-dependent functions, such as intra-S checkpoint, and IR-induced apoptosis.

Finally, as proposed in Specific Aim 3, we are collaborating with Dr. Wanguo Liu in screening MDC1expression mutations in breast cancer patients.

Key Research Accomplishments

BRCA1, a downstream effector of Chk2 and MDC1, interacts with topoisomerase II.

BRCA1 maintains genomic stability through regulating DNA decatenation.

MDC1 interacts with DNA-PKcs/Ku through the repeat region of MDC1

MDC1 regulates DNA-PKcs phosphorylation and localization.

MDC1 plays an important role in DNA repair regulated by DNA-PK.

Generation of an animal model to evaluate the in vivo function of MDC1.

$MDC1^{-/-}$ mice are growth retarded.

$MDC1^{-/-}$ mice are hypersensitive to radiaton.

$MDC1^{-/-}$ mice show spontaneous genomic instability.

Reportable Outcomes (Aug. 2004-Aug. 2005)

Publications:

1. Lou, Z, Chen, B. P-C., Asaithamby, A., Minter-Dykhouse, K., Chen, D.J. and Chen, J. MDC1 Regulates DNA-PK Autophosphorylation in Response to DNA Damage. ***J. Biol. Chem.*** **279**:46359-62, 2004.

2. Lou,Z and Junjie Chen. BRCA1 Participates In DNA Decatenation. ***Nature Structure and Molecular Biology,* 12:589-563, 2005.**

3. Lou, Z and Chen, J. Mammalian DNA Damage Response Pathway. ***In Press. Genome Instability in Cancer Development. Springer Press***

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Selected for oral presentation at American Association of Cancer Research, April 2005

Era of Hope, Jun, 2005

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N/A

Conclusions

We characterized one mechanism how BRCA1, a downstream effector of MDC1 and Chk2, in maintaining genomic stability. We show that BRCA1 interacts with topoisomerase II and regulates topoisomerase II activity and localization. In the absence of BRCA1, cells are defective in chromosome segregation, which might account for genomic instability in BRCA1-deficient cells. We also characterized the interaction between MDC1 and DNA-PKcs/Ku, a upstream regulator of Chk2. We show that MDC1 regulates DNA-PKcs phosphorylation and localization, and participates in DNA repair. Furthermore, we generated $MDC1^{-/-}$ mice and in the process of evaluating the physiological function of MDC1. Our preliminary data suggest that MDC1 plays an important role in DNA damage response and maintaining genomic stability. We will continue work on Specific Aim 3: to screen human tumor cell line for MDC1 mutations. In summery, our studies revealed novel mechanisms of the DNA damage response pathway and will provide new insight into the maintenance of genomic stability. Animal models with knockout mice will elucidate how dysfunction of the Chk2-MDC1 pathway contributes to the development of tumor.

References

Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J. N., Ried, T., Tagle, D., and Wynshaw-Boris, A. (1996). Atm-deficient mice: a paradigm of ataxia telangiectasia. Cell *86*, 159-171.

Bell, D. W., Varley, J. M., Szydlo, T. E., Kang, D. H., Wahrer, D. C., Shannon, K. E., Lubratovich, M., Verselis, S. J., Isselbacher, K. J., Fraumeni, J. F., *et al.* (1999). Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science *286*, 2528-2531.

Celeste, A., Petersen, S., Romanienko, P. J., Fernandez-Capetillo, O., Chen, H. T., Sedelnikova, O. A., Reina-San-Martin, B., Coppola, V., Meffre, E., Difilippantonio, M. J., *et al.* (2002). Genomic instability in mice lacking histone H2AX. Science *296*, 922-927.

Elson, A., Wang, Y., Daugherty, C. J., Morton, C. C., Zhou, F., Campos-Torres, J., and Leder, P. (1996). Pleiotropic defects in ataxia-telangiectasia protein-deficient mice. Proc Natl Acad Sci U S A *93*, 13084-13089.

Goldberg, M., Stucki, M., Falck, J., D'Amours, D., Rahman, D., Pappin, D., Bartek, J., and Jackson, S. P. (2003). MDC1 is required for the intra-S-phase DNA damage checkpoint. Nature *421*, 952-956.

Lou, Z., Chini, C. C., Minter-Dykhouse, K., and Chen, J. (2003a). Mediator of DNA damage checkpoint protein 1 regulates BRCA1 localization and phosphorylation in DNA damage checkpoint control. J Biol Chem *278*, 13599-13602.

Lou, Z., Minter-Dykhouse, K., Wu, X., and Chen, J. (2003b). MDC1 is coupled to activated CHK2 in mammalian DNA damage response pathways. Nature *421*, 957-961.

Meijers-Heijboer, H., van den Ouweland, A., Klijn, J., Wasielewski, M., de Snoo, A., Oldenburg, R., Hollestelle, A., Houben, M., Crepin, E., van Veghel-Plandsoen, M., *et al.* (2002). Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations. Nat Genet *31*, 55-59.

Peng, A., and Chen, P. L. (2003). NFBD1, like 53BP1, is an early and redundant transducer mediating Chk2 phosphorylation in response to DNA damage. J Biol Chem *278*, 8873-8876.

Scully, R., and Livingston, D. M. (2000). In search of the tumour-suppressor functions of BRCA1 and BRCA2. Nature *408*, 429-432.

Shang, Y., Bodero, A., and Chen, P. (2002). NFBD1, a novel nuclear protein with signature motifs of FHA and BRCT, and an internal 41 amino acid repeat sequence, is an early participant in DNA damage response. J Biol Chem *278*, 6323-6329.

Stewart, G. S., Wang, B., Bignell, C. R., Taylor, A. M., and Elledge, S. J. (2003). MDC1 is a mediator of the mammalian DNA damage checkpoint. Nature *421*, 961-966.

Vahteristo, P., Bartkova, J., Eerola, H., Syrjakoski, K., Ojala, S., Kilpivaara, O., Tamminen, A., Kononen, J., Aittomaki, K., Heikkila, P., *et al.* (2002). A CHEK2 genetic variant contributing to a substantial fraction of familial breast cancer. Am J Hum Genet *71*, 432-438.

Xu, X., and Stern, D. F. (2002). NFBD1/KIAA0170 is a chromatin-associated protein involved in DNA damage signaling pathways. J Biol Chem *278*, 8795-8803.

Xu, X., and Stern, D. F. (2003). NFBD1/MDC1 regulates ionizing radiation-induced focus formation by DNA checkpoint signaling and repair factors. Faseb J *17*, 1842-1848.

Xu, Y., Ashley, T., Brainerd, E. E., Bronson, R. T., Meyn, M. S., and Baltimore, D. (1996). Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma. Genes Dev *10*, 2411-2422.

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BRCA1 participates in DNA decatenation

Zhenkun Lou, Katherine Minter-Dykhouse & Junjie Chen

The tumor suppressor BRCA1 has an important function in the maintenance of genomic stability. Increasing evidence suggests that BRCA1 regulates cell cycle checkpoints and DNA repair after DNA damage. However, little is known about its normal function in the absence of DNA damage. Here we show that BRCA1 interacts and colocalizes with topoisomerase IIα in S phase cells. Similar to cells treated with the topoisomerase IIα inhibitor ICRF-193, BRCA1-deficient cells show lagging chromosomes, indicating a defect in DNA decatenation and chromosome segregation. More directly, BRCA1 deficiency results in defective DNA decatenation *in vitro*. Finally, topoisomerase IIα is ubiquitinated in a BRCA1-dependent manner, and topoisomerase IIα ubiquitination correlates with higher DNA decatenation activity. Together these results suggest an important role of BRCA1 in DNA decatenation and reveal a previously unknown function of BRCA1 in the maintenance of genomic stability.

BRCA1 is important in the maintenance of genomic integrity. In response to DNA damage, BRCA1 participates in many aspects of the DNA damage–signaling pathway, including intra-S checkpoint, G2/M checkpoint and DNA repair through homologous recombination[1–3]. BRCA1 knockout mice are embryonically lethal[4], suggesting an essential role for BRCA1 during embryonic development. Cells derived from *BRCA1*$^{\Delta 11/\Delta 11}$ or conditional knockout mice have various chromosome abnormalities[5,6], confirming the importance of BRCA1 in the maintenance of genomic stability. In S phase cells, BRCA1 localizes to nuclear foci[7,8], and some but not all of these S phase BRCA1 foci colocalize with proliferating cell nuclear antigen (PCNA). The colocalization of BRCA1 and PCNA becomes apparent after DNA replication stress, supporting a role for BRCA1 in damage-induced S phase checkpoint control[7]. However, the role of BRCA1 in normal S phase remains unclear. The partial colocalization of BRCA1 with replication foci raises the possibility that BRCA1 carries out certain functions in S phase, but these functions may not be directly involved in DNA replication per se. Previous studies have shown that, much like BRCA1, topoisomerase IIα also forms nuclear foci in S phase. Again, some but not all of these foci colocalize with BrdU$^+$ replication foci[9,10]. The similar behavior of BRCA1 and topoisomerase IIα in normal S phase prompted us to examine the potential connection between these two proteins.

RESULTS

BRCA1 interacts with topoisomerase IIα

We first examined the localization of BRCA1 and topoisomerase IIα in S phase cells by immunofluorescence staining. We found that BRCA1 colocalized with topoisomerase IIα in S phase foci (**Fig. 1a**). These findings led us to test whether BRCA1 interacts with topoisomerase IIα *in vivo*. Using BRCA1-deficient HCC1937 cells and HCC1937 cells reconstituted with BRCA1 (HCCBRCA1), we found that only wild-type BRCA1 immunoprecipitated with topoisomerase IIα (**Fig. 1b,c**). Although HCC1937 cells still express a mutant form of BRCA1 with C-terminus truncation, we did not detect any interaction between topoisomerase IIα and this truncated BRCA1, suggesting that the C-terminal part of BRCA1 is required for interaction between BRCA1 and topoisomerase IIα. BRCA1 contains tandem BRCT domains at its C terminus, which binds phospho-Ser/Thr motifs[11,12]. To test if the BRCT domains of BRCA1 are involved in the interaction with topoisomerase IIα, we used GST-BRCA1 BRCT domains to immunoprecipitate topoisomerase IIα from cell lysates treated with or without α-phosphatase. The BRCT domains of BRCA1 bound topoisomerase IIα in a phosphorylation-dependent manner, again supporting interaction between BRCA1 and topoisomerase IIα interaction (**Fig. 1d**). The interaction and colocalization of BRCA1 with topoisomerase IIα led us to investigate further the role of BRCA1 in functions dependent on topoisomerase IIα.

BRCA1 and chromosome mis-segregation

Topoisomerase IIα is essential for chromosome decatenation after DNA replication[13]. Treatment of HeLa cells with ICRF-193 (2 μM), an inhibitor of topoisomerase IIα[14,15], resulted in a defect in chromosome segregation, characteristically demonstrated by lagging chromosomes forming bridgelike structures during mitosis (**Fig. 2a**). This defect is probably due to failure of proper chromosome decatenation after DNA replication. Notably, in BRCA1-deficient HCC1937 cells, we often observed cells with lagging chromosomes, similar to the cells treated with ICRF-193 (**Fig. 2a**). About 11% of unsynchronized HCC1937 cells showed lagging chromosomes (**Fig. 2b**). In contrast, in HCC1937 cells reconstituted with BRCA1, we detected only a few cells (<0.7%) with lagging chromosomes (**Fig. 2b**). We did not observe an obvious difference in overall cell cycle profile between HCC1937 and HCCBRCA1 cells (data not shown).

To confirm further that the defective chromosome segregation observed in HCC1937 cells is due to the loss of BRCA1, we used short interfering RNA (siRNA) to downregulate BRCA1 in HeLa cells

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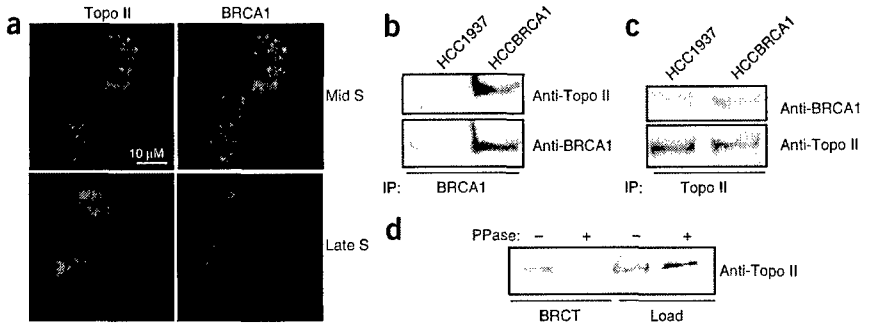

Figure 1 BRCA1 colocalizes and interacts with topoisomerase IIα. (**a**) HeLa cells were immunostained with the indicated antibodies. Representative images of S phase cells are shown. (**b**,**c**) Topoisomerase IIα or BRCA1 was immunoprecipitated (IP) from HCC1937 (BRCA1-deficient) cells or HCCBRCA1 cells and blotted with the indicated antibodies. (**d**) GST-BRCA1 BRCT domain was used to immunoprecipitate topoisomerase IIα from HeLa cell lysates treated either with buffer or λ-phosphatase (PPase). Anti-Topo II, antibody to topoisomerase IIα; Anti-BRCA1, antibody to BRCA1.

(**Fig. 2c**). Knockdown of BRCA1 did not affect topoisomerase IIα expression and vice versa. Again, there was no difference in overall cell cycle profile for cells transfected with control siRNA or BRCA1 siRNA (data not shown). Cells transfected with control siRNA have normal chromosome segregation (**Fig. 2b**). In contrast, cells transfected with BRCA1 siRNA show a dramatic defect in chromosome segregation, with 10% of cells showing lagging chromosomes, similar to that of cells transfected with topoisomerase IIα siRNA (**Fig. 2a,b**). These results suggest that loss of BRCA1 results in defective chromosome segregation during mitosis, a phenomenon similar to that observed in cells deficient in topoisomerase IIα.

It is possible that BRCA1's function in DNA damage checkpoints could contribute to defective chromosome segregation. However, we did not observe any chromosome segregation defects in HeLa cells transfected with Chk1 siRNA (data not shown). Because Chk1 is an important downstream effector of BRCA1 in the intra-S and G2/M checkpoint after DNA damage[16], it is improbable that defects in DNA damage checkpoint activation in BRCA1-deficient cells would contribute to the flawed chromosome segregation. Another possible explanation for defective chromosome segregation is that BRCA1 may function in the mitotic checkpoint. However, we found that the mitotic checkpoint activation after nocodazole treatment is intact in HCC1937 cells (**Fig. 3a**), arguing against a direct role of BRCA1 in mitotic checkpoint control. In addition, we did not observe in BRCA1-deficient cells premature sister chromatid separation (data not shown), which is a hallmark of a defective mitotic checkpoint. Notably, we observed a high percentage of elongated and tangled chromosomes from HCC1937 cells, similar to those of cells treated with ICRF-193 (**Fig. 3b,c**). Downregulation of topoisomerase IIα or BRCA1 using siRNA also resulted in a high percentage of elongated and tangled chromosomes (**Fig. 3b,d**). These results further suggest that BRCA1 might have a direct effect on chromosome decatenation and subsequently affect chromosome condensation.

BRCA1 is involved in DNA decatenation

The major function of topoisomerase IIα, that of separating catenated chromosomes after DNA replication, is required for efficient chromosome condensation. Topoisomerase IIα has been shown to decatenate replicated DNA behind replication forks[17]. Because we observed S phase colocalization of BRCA1 with topoisomerase IIα, and defective chromosome segregation in BRCA1-deficient cells, we next examined whether BRCA1 could be involved in DNA decatenation. Early reports have established that nuclear extracts from cells can decatenate kinetoplast DNA (kDNA) in a topoisomerase IIα–dependent manner *in vitro*[18]. We also found that the decatenation activity was completely blocked when HeLa nuclear extracts were pretreated with ICRF-193 (data not shown), suggesting that DNA decatenation is mediated chiefly by topoisomerase IIα.

We next asked whether BRCA1 also participates in DNA decatenation *in vitro*. Nuclear extracts were prepared from HCC1937 or HCCBRCA1 cells and used in the *in vitro* decatenation assays. Nuclear extracts from HCCBRCA1 cells efficiently decatenated kDNA within 10 min (**Fig. 4a**). In contrast, nuclear extracts isolated from HCC1937 cells were less efficient in decatenating kDNA (**Fig. 4a**). The decreased decatenation activity in HCC1937 nuclear extracts was not due to a decrease in topoisomerase IIα concentrations, because nuclear extracts from HCC1937 and HCCBRCA1 cells contained equal amounts of topoisomerase IIα (**Fig. 4a**, lower panels). To confirm further the role

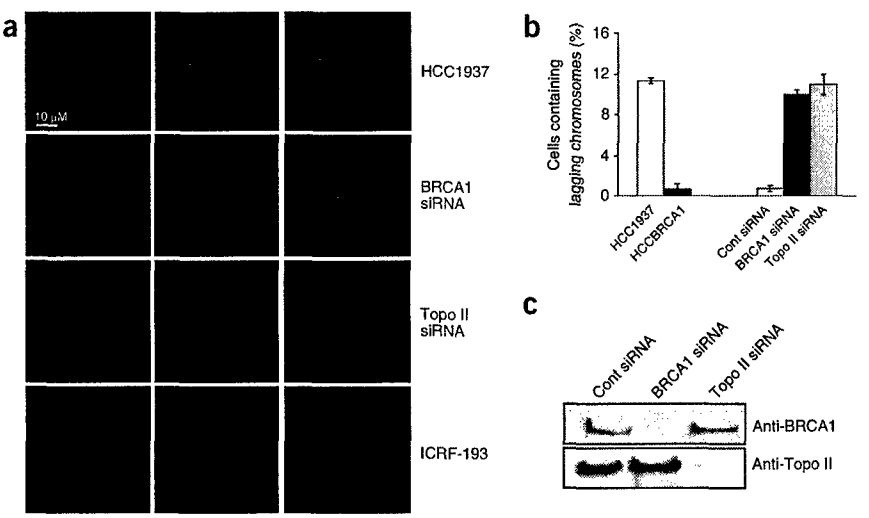

Figure 2 Absence of BRCA1 results in defective chromosome segregation. (**a**) HeLa cells were transfected with BRCA1 siRNA or topoisomerase IIα siRNA, fixed and stained with DAPI. Similarly, HCC1937 cells or HeLa cells treated with 2 μM ICRF-193 for 1 h were also stained with DAPI. Representative examples of defective chromosome segregation in these samples were shown. (**b**) Quantification of cells with defective chromosome segregation. The *y*-axis represents the percentage of cells with lagging chromosomes. (**c**) Western blotting was done to demonstrate the downregulation of BRCA1 or topoisomerase IIα by siRNA transfection. Anti-Topo II, antibody to topoisomerase IIα; Anti-BRCA1, antibody to BRCA1; Cont, control.

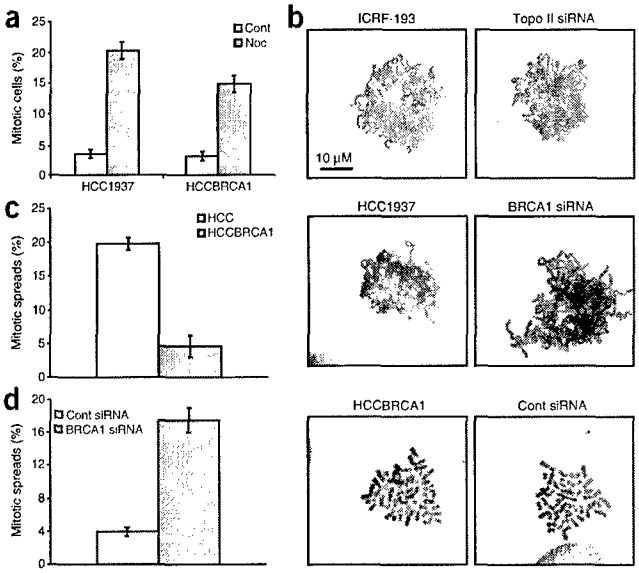

Figure 3 Absence of BRCA1 results in defective chromosome condensation. (**a**) HCC1937 or HCCBRCA1 cells were treated with 0.2 μg ml^{-1} nocodazole (Noc). Cells were evaluated 12 h later for anti-phospho H3 population. The *y*-axis represents percentage of mitotic cells. (**b**) Representative examples of mitotic spreads from HCC1937, HCCBRCA1, HCCBRCA1 treated with ICRF-193 (2 μM) and caffeine (7 mM), or HeLa cells transfected with indicated siRNAs. (**c**,**d**) Quantification of mitotic spreads with undercondensed chromosomes from **b**. The *y*-axis represents percentage of mitotic spreads with undercondensed chromosomes. Cont, Control; Topo II, topoisomerase IIα.

of BRCA1 in DNA decatenation, we used siRNA to downregulate BRCA1 in HeLa cells. We prepared nuclear extracts from cells transfected with either control siRNA or BRCA1 siRNA and carried out the *in vitro* decatenation assay. We observed less decatenation activity in nuclear extracts from cells transfected with BRCA1 siRNA than in those from cells transfected with control siRNA (**Fig. 4b**). Topoisomerase IIα concentrations were equivalent in the control sample and sample treated with BRCA1 siRNA (**Fig. 4b**, lower panels). To measure decatenation activity quantitatively, we used different amounts of nuclear extracts from HCC1937 or HCCBRCA1 cells, and conducted the *in vitro* decatenation assay. Nuclear extracts from HCCBRCA1 cell had about three times more decatenation activity than did extracts from HCC1937 cells (**Fig. 4c,d**), even though both contain equivalent amounts of topoisomerase IIα (**Fig. 4e**). Collectively, these assays suggest a role for BRCA1 in DNA decatenation. Therefore, the defect in chromosome segregation observed in BRCA1-deficient cells is likely to be due to the failure of proper DNA decatenation after DNA replication.

BRCA1 regulates topoisomerase IIα ubiquitination

Although our assays suggest a role of BRCA1 in DNA decatenation, it is not clear how BRCA1 regulates DNA decatenation *in vivo*. Phosphorylation of topoisomerase IIα has been linked to the regulation of its activities[19]. We next studied whether BRCA1 would regulate topoisomerase IIα phosphorylation. Topoisomerase IIα was immunoprecipitated from HCC1937 or HCCBRCA1 cells and blotted with antibodies to the mitotic phosphoprotein MPM-2, which has been shown to recognize phosphorylated topoisomerase IIα (ref. 20). BRCA1 deficiency did not affect topoisomerase IIα phosphorylation (**Fig. 5a**).

Because BRCA1-BARD1 has E3 ubiquitin ligase activity[21,22], it is possible BRCA1 may modulate topoisomerase IIα through ubiquitination. When topoisomerase IIα was immunoprecipitated from HCCBRCA1 cells, we observed smears with slower mobility, which could also be detected by antibodies to ubiquitin (**Fig. 5b**). No smear was detected in topoisomerase IIα immunoprecipitated from HCC1937 cells (**Fig. 5b**), suggesting that BRCA1-dependent ubiquitination of topoisomerase IIα occurred. To confirm further that BRCA1 is required for topoisomerase IIα ubiquitination, we used siRNA to downregulate BRCA1. We observed smears with slower mobility in topoisomerase IIα immunoprecipitates from HeLa cells transfected with control siRNA, which could also be detected by antibodies to ubiquitin (**Fig. 5c**). However, transfection with BRCA1 siRNA or

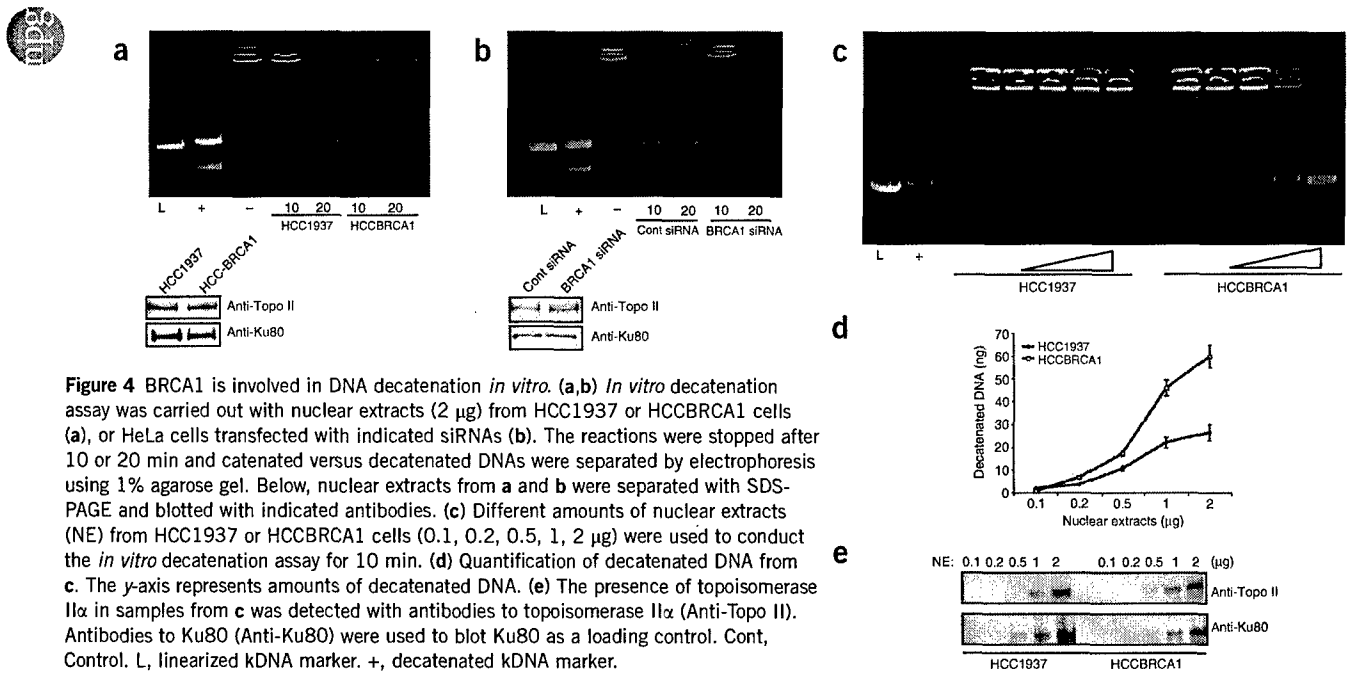

Figure 4 BRCA1 is involved in DNA decatenation *in vitro*. (**a**,**b**) *In vitro* decatenation assay was carried out with nuclear extracts (2 μg) from HCC1937 or HCCBRCA1 cells (**a**), or HeLa cells transfected with indicated siRNAs (**b**). The reactions were stopped after 10 or 20 min and catenated versus decatenated DNAs were separated by electrophoresis using 1% agarose gel. Below, nuclear extracts from **a** and **b** were separated with SDS-PAGE and blotted with indicated antibodies. (**c**) Different amounts of nuclear extracts (NE) from HCC1937 or HCCBRCA1 cells (0.1, 0.2, 0.5, 1, 2 μg) were used to conduct the *in vitro* decatenation assay for 10 min. (**d**) Quantification of decatenated DNA from **c**. The *y*-axis represents amounts of decatenated DNA. (**e**) The presence of topoisomerase IIα in samples from **c** was detected with antibodies to topoisomerase IIα (Anti-Topo II). Antibodies to Ku80 (Anti-Ku80) were used to blot Ku80 as a loading control. Cont, Control. L, linearized kDNA marker. +, decatenated kDNA marker.

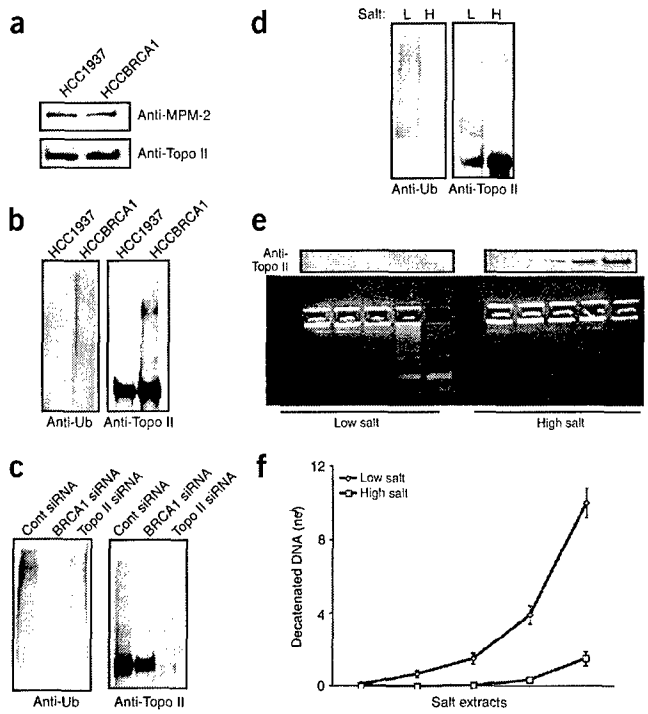

Figure 5 Topoisomerase IIα (Topo II) is ubiquitinated in a BRCA1-dependent manner. (**a**, **b**) HCC1937 and HCCBRCA1 cells were lysed, and Topo II was immunoprecipitated and blotted with the indicated antibodies. Anti-MPM-2, Antibodies to MPM-2; Anti-Topo II, antibodies to Topo II; Anti-Ub, antibodies to ubiquitin. (**c**) HeLa cells were transfected with indicated siRNAs, and Topo II was immunoprecipitated and blotted with the indicated antibodies. Cont, Control. (**d**) HeLa cells were sequentially extracted with low-salt (L, 150 mM NaCl) and high-salt (H, 350 mM NaCl) extraction buffers. Topo II was immunoprecipitated and blotted with the indicated antibodies. (**e**) Different amounts of Topo II isolated in **d** were used for the *in vitro* decatenation assay. Topo II present in each sample was blotted with antibodies to Topo II. (**f**) Quantification of decatenated DNA in samples from **e**.

topoisomerase IIα siRNA resulted in the disappearance or decrease of the smears detected by antibodies to ubiquitin (**Fig. 5c**), further supporting the idea that topoisomerase IIα is ubiquitinated in a BRCA1-dependent manner *in vivo*. Topoisomerase IIα ubiquitination might be involved in topoisomerase IIα degradation[23]. However, the BRCA1-dependent topoisomerase IIα polyubiquitination did not seem to affect the steady-state topoisomerase IIα levels (**Figs. 1a**, **4a**,**b**,**e** and **5a–c**). Pulse-chase analysis also did not demonstrate enhanced degradation of topoisomerase IIα in BRCA1-proficient cells (data not shown). Therefore, it is improbable that BRCA1-dependent topoisomerase IIα ubiquitination contributes to topoisomerase IIα degradation.

In yeast and *Xenopus laevis* systems, modification of topoisomerase IIα by sumolation has been linked to altered chromatin binding dynamics of topoisomerase[24,25]. However, we did not detect apparent topoisomerase IIα sumolation in human cells (data not shown). We then examined whether the ubiquitination of topoisomerase IIα could modulate its chromatin binding *in vivo*. We used different concentrations of salts to study the extractability of topoisomerase IIα. HeLa cells were subjected to low-salt extraction (150 mM NaCl) followed by high-salt extraction (350 mM NaCl). Topoisomerase IIα was then immunoprecipitated from the low-salt and high-salt extracted fractions and blotted with antibodies to ubiquitin or to topoisomerase IIα. Notably, most of the ubiquitinated topoisomerase IIα was detected at low-salt fractions, although the majority of unmodified topoisomerase IIα existed in high-salt fractions (**Fig. 5d**). These results suggest that the BRCA1-dependent ubiquitination of topoisomerase IIα may affect the mobility of topoisomerase IIα *in vivo*. At the present time, we could not directly evaluate how BRCA1-dependent ubiquitination of topoisomerase IIα affects topoisomerase IIα activities, because the ubiquitination sites on topoisomerase IIα are unknown. However, previous studies have shown that topoisomerase IIα stably bound to chromatin has less enzymatic activity[26]. Indeed, we observed that topoisomerase IIα from low-salt fractions showed higher activities when evaluated by the *in vitro* decatenation assays (**Fig. 5e**,**f**), even though more topoisomerase IIα is present in high-salt fractions (**Fig. 5e**). The difference in decatenation activities was not due to different salt concentration in the buffer, because topoisomerase IIα isolated from both fractions was resuspended in the same buffer. Therefore, there is an apparent correlation between BRCA1-dependent topoisomerase IIα ubiquitination and higher topoisomerase IIα activities, suggesting that BRCA1 might regulate topoisomerase IIα distribution and activity through ubiquitination.

DISCUSSION

Here we show that BRCA1 participates in topoisomerase IIα–dependent DNA decatenation. One potential mechanism by which BRCA1 regulates DNA decatenation is through the ubiquitination of topoisomerase IIα, which affects topoisomerase IIα distribution and activity. These findings reveal a novel function of BRCA1 in the maintenance of genomic stability.

BRCA1 interacts with and colocalizes with topoisomerase IIα during S phase. In addition, our data suggest that BRCA1 binds phosphorylated topoisomerase IIα through its BRCT domains (**Fig. 1d**). It will be instructive to identify the phosphorylation sites on topoisomerase IIα that are recognized by the BRCA1 BRCT domains. We speculate that the phosphorylation of topoisomerase IIα at these sites results in the binding of BRCA1-BARD1 and thus facilitates the ubiquitination of topoisomerase IIα. Topoisomerase IIα is polyubiquitinated *in vivo* in a BRCA1-dependent manner (**Fig. 5a–c**). However, we did not detect efficient topoisomerase IIα ubiquitination *in vitro*. So far, the *in vitro* ubiquitination assay that depends on BRCA1-BARD1 is not very sensitive. Other cofactors may be required for the maximum efficiency of these reactions *in vivo*. It is also possible that the purified topoisomerase IIα we used was not phosphorylated at the sites recognized by the BRCA1 BRCT domain and thus could not be used as a proper substrate in these reactions. Further experiments are needed to address these issues.

The S phase localization (foci) of BRCA1 and topoisomerase IIα underlines their role in DNA decatenation after DNA replication. In the absence of BRCA1, there is only one-third as much DNA decatenation activity (**Fig. 4**)—a situation that may contribute to the observed defect in chromosome segregation in BRCA1-deficient cells (**Figs. 2** and **3**). The chromosome segregation defect has also been reported in $BRCA1^{\Delta 11/\Delta 11}$ mouse embryonic fibroblasts[27], and the defect in spindle checkpoint has been proposed as the underlying mechanism. However, we did not detect any obvious defect in the mitotic checkpoint in HCC1937 cells (**Fig. 3a**) or HeLa cells transfected with BRCA1 siRNA (data not shown). It is not clear what accounts for these differences. One possibility is that the $BRCA1^{\Delta 11/\Delta 11}$ isoform may have a dominant negative effect on the mitotic checkpoint.

We propose that one new mechanism by which BRCA1 regulates genomic stability is by participating in DNA decatenation. This normal S phase function of BRCA1, together with BRCA1's check-

point function, helps to guard genomic stability. BRCA1-deficient cells have many spontaneous chromosome abnormalities[5,6]. The chromosome abnormities observed in BRCA1-deficient cells might be caused by defective DNA decatenation. Previous studies have shown that prolonged treatment of cells with the non-DNA-damaging topoisomerase IIα inhibitor ICRF-193 resulted in chromosome gaps and recombination, because cells were able to override the decatenation checkpoint[28]. It has also been suggested that BRCA1 is involved in the decatenation checkpoint[29]. Therefore, in BRCA1-deficient cells, inefficient DNA decatenation together with defective decatenation checkpoint would result in forced segregation of still-catenated chromosomes, causing chromosome breaks and recombination.

In conclusion, we have identified a heretofore unknown function of BRCA1 in DNA decatenation. These findings help us understand how BRCA1 maintains genomic stability—not only by activating DNA damage checkpoint, but also by regulating chromosome segregation.

METHODS

Antibodies and reagents. Antibodies to BRCA1 (MS110 and SD118) were generated as described[30,31]. Antibodies to topoisomerase IIα for immunofluorescence staining and immunoblotting were obtained from NeoMarkers. Antibodies to topoisomerase IIα for immunoprecipitation were obtained from Bethyl Laboratories. Antibodies to ubiquitin were obtained from Zymed. Antibodies to MPM-2 and to Ku80 were obtained from Upstate. Antibodies to phospho-H3 were obtained from Cell Signaling. ICRF-193 was purchased from MP Biomedicals. Nocodazole was obtained from Sigma and colcemid from Gibco.

Immunoprecipitation, immunoblotting and immunostaining experiments. Cell lysate preparation, immunoprecipitation, immunoblotting and immunostaining were performed as described[7,32]. For topoisomerase IIα ubiquitination *in vivo*, 10×10^6 cells were lysed in 100 μl 1% (w/v) SDS, then diluted with 900 μl NETN buffer composed of 0.5% (v/v) Nonidet P-40 (NP-40), 1 mM EDTA, 50 mM Tris, 100 mM NaCl, pH 7.4, with protease inhibitor cocktail, and immunoprecipitated with antibodies to topoisomerase IIα.

siRNA transfection. All siRNAs were synthesized by Dharmacon. The sequence for control siRNA, MDC1 siRNA and BRCA1 siRNA was described[33,34]. The sequence for topoisomerase IIα siRNA corresponded to topoisomerase IIα cDNA 76-96. Cells were transfected with indicated siRNAs as described[34].

Mitotic spread preparation. Mitotic spreads were prepared using standard protocol. Cells were treated with 25 ng ml^{-1} colcemid for 2 h, then treated with 0.075 M KCl hypotonic solution for 20 min at 37 °C. Cells were then fixed with 3:1 (v/v) methanol/acetic acid, and aliquots of cell suspension were dropped to vaporized slides. Dried slides were stained with Giemsa (Gibco).

***In vitro* decatenation assay.** *In vitro* decatenation assay kit was purchased from Topogen, and the assays were performed as suggested by the manufacturer.

Cell extraction. Cells grown on a 100-mm plate were rinsed with cold PBS once, and extracted with NETN buffer (0.5% (v/v) NP-40, 1 mM EDTA, 50 mM Tris, with 150 mM NaCl) on ice for 10 min. The extracts were collected, and what remained on the plate was extracted again with NETN buffer containing 350 mM NaCl on ice for 10 min.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Venkitaraman, A.R. Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* **108**, 171–182 (2002).
2. Scully, R. & Livingston, D.M. In search of the tumour-suppressor functions of BRCA1 and BRCA2. *Nature* **408**, 429–432 (2000).
3. Kerr, P. & Ashworth, A. New complexities for BRCA1 and BRCA2. *Curr. Biol.* **11**, R668–R676 (2001).
4. Brodie, S.G. & Deng, C.X. BRCA1-associated tumorigenesis: what have we learned from knockout mice? *Trends Genet.* **17**, S18–S22 (2001).
5. Xu, X. *et al.* Genetic interactions between tumor suppressors Brca1 and p53 in apoptosis, cell cycle and tumorigenesis. *Nat. Genet.* **28**, 266–271 (2001).
6. Xu, X. *et al.* Conditional mutation of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. *Nat. Genet.* **22**, 37–43 (1999).
7. Scully, R. *et al.* Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. *Cell* **90**, 425–435 (1997).
8. Scully, R. *et al.* Association of BRCA1 with Rad51 in mitotic and meiotic cells. *Cell* **88**, 265–275 (1997).
9. Agostinho, M. *et al.* Human topoisomerase IIalpha: targeting to subchromosomal sites of activity during interphase and mitosis. *Mol. Biol. Cell* **15**, 2388–2400 (2004).
10. Niimi, A., Suka, N., Harata, M., Kikuchi, A. & Mizuno, S. Co-localization of chicken DNA topoisomerase IIalpha, but not beta, with sites of DNA replication and possible involvement of a C-terminal region of alpha through its binding to PCNA. *Chromosoma* **110**, 102–114 (2001).
11. Yu, X., Chini, C.C., He, M., Mer, G. & Chen, J. The BRCT domain is a phospho-protein binding domain. *Science* **302**, 639–642 (2003).
12. Manke, I.A., Lowery, D.M., Nguyen, A. & Yaffe, M.B. BRCT repeats as phosphopeptide-binding modules involved in protein targeting. *Science* **302**, 636–639 (2003).
13. Wang, J.C. DNA topoisomerases. *Annu. Rev. Biochem.* **65**, 635–692 (1996).
14. Ishida, R. *et al.* Inhibition of intracellular topoisomerase II by antitumor bis(2,6-dioxopiperazine) derivatives: mode of cell growth inhibition distinct from that of cleavable complex-forming type inhibitors. *Cancer Res.* **51**, 4909–4916 (1991).
15. Clarke, D.J., Johnson, R.T. & Downes, C.S. Topoisomerase II inhibition prevents anaphase chromatid segregation in mammalian cells independently of the generation of DNA strand breaks. *J. Cell Sci.* **105**, 563–569 (1993).
16. Yarden, R.I., Pardo-Reoyo, S., Sgagias, M., Cowan, K.H. & Brody, L.C. BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage. *Nat. Genet.* **30**, 285–289 (2002).
17. Lucas, I., Germe, T., Chevrier-Miller, M. & Hyrien, O. Topoisomerase II can unlink replicating DNA by precatenane removal. *EMBO J.* **20**, 6509–6519 (2001).
18. Marini, J.C., Miller, K.G. & Englund, P.T. Decatenation of kinetoplast DNA by topoisomerases. *J. Biol. Chem.* **255**, 4976–4979 (1980).
19. Poljak, L. & Kas, E. Resolving the role of topoisomerase II in chromatin structure and function. *Trends Cell Biol.* **5**, 348–354 (1995).
20. Taagepera, S., Rao, P.N., Drake, F.H. & Gorbsky, G.J. DNA topoisomerase II alpha is the major chromosome protein recognized by the mitotic phosphoprotein antibody MPM-2. *Proc. Natl Acad. Sci. USA* **90**, 8407–8411 (1993).
21. Baer, R. & Ludwig, T. The BRCA1/BARD1 heterodimer, a tumor suppressor complex with ubiquitin E3 ligase activity. *Curr. Opin. Genet. Dev.* **12**, 86–91 (2002).
22. Starita, L.M. & Parvin, J.D. The multiple nuclear functions of BRCA1: transcription, ubiquitination and DNA repair. *Curr. Opin. Cell Biol.* **15**, 345–350 (2003).
23. Salmena, L., Lam, V., McPherson, J.P. & Goldenberg, G.J. Role of proteasomal degradation in the cell cycle-dependent regulation of DNA topoisomerase IIalpha expression. *Biochem. Pharmacol.* **61**, 795–802 (2001).
24. Bachant, J., Alcasabas, A., Blat, Y., Kleckner, N. & Elledge, S.J. The SUMO-1 isopeptidase Smt4 is linked to centromeric cohesion through SUMO-1 modification of DNA topoisomerase II. *Mol. Cell* **9**, 1169–1182 (2002).
25. Azuma, Y., Arnaoutov, A. & Dasso, M. SUMO-2/3 regulates topoisomerase II in mitosis. *J. Cell Biol.* **163**, 477–487 (2003).
26. Meyer, K.N. *et al.* Cell cycle-coupled relocation of types I and II topoisomerases and modulation of catalytic enzyme activities. *J. Cell Biol.* **136**, 775–788 (1997).
27. Wang, R.H., Yu, H. & Deng, C.X. A requirement for breast-cancer-associated gene 1 (BRCA1) in the spindle checkpoint. *Proc. Natl Acad. Sci. USA* **101**, 17108–17113 (2004).
28. Gimenez-Abian, J.F. *et al.* Premitotic chromosome individualization in mammalian cells depends on topoisomerase II activity. *Chromosoma* **109**, 235–244 (2000).
29. Deming, P.B. *et al.* The human decatenation checkpoint. *Proc. Natl Acad. Sci. USA* **98**, 12044–12049 (2001).
30. Scully, R. *et al.* Location of BRCA1 in human breast and ovarian cancer cells. *Science* **272**, 123–126 (1996).
31. Chen, J.J., Silver, D., Cantor, S., Livingston, D.M. & Scully, R. BRCA1, BRCA2, and Rad51 operate in a common DNA damage response pathway. *Cancer Res.* **59**, 1752s–1756s (1999).
32. Rappold, I., Iwabuchi, K., Date, T. & Chen, J. Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. *J. Cell Biol.* **153**, 613–620 (2001).
33. Lou, Z., Minter-Dykhouse, K., Wu, X. & Chen, J. MDC1 is coupled to activated CHK2 in mammalian DNA damage response pathways. *Nature* **421**, 957–961 (2003).
34. Lou, Z., Chini, C.C., Minter-Dykhouse, K. & Chen, J. Mediator of DNA damage checkpoint protein 1 regulates BRCA1 localization and phosphorylation in DNA damage checkpoint control. *J. Biol. Chem.* **278**, 13599–13602 (2003).

Accelerated Publication

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MDC1 Regulates DNA-PK Autophosphorylation in Response to DNA Damage*[S]

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DNA damage initiates signaling events through kinase cascades that result in cell cycle checkpoint control and DNA repair. However, it is not yet clear how the signaling pathways relay to DNA damage repair. Using the repeat region of checkpoint protein MDC1 (mediator of DNA damage checkpoint protein 1), we identified DNA-PKcs/Ku as MDC1-associated proteins. Here, we show that MDC1 directly interacts with the Ku/DNA-PKcs complex. Down-regulation of MDC1 resulted in defective phospho-DNA-PKcs foci formation and DNA-PKcs autophosphorylation, suggesting that MDC1 regulates autophosphorylation of DNA-PKcs following DNA damage. Furthermore, DNA-PK-dependent DNA damage repair is defective in cells depleted of MDC1. Taken together, these results suggest that the MDC1 repeat region is involved in protein-protein interaction with DNA-PKcs/Ku, and MDC1 regulates DNA damage repair by influencing DNA-PK autophosphorylation. Therefore, MDC1 acts not only as a mediator of DNA damage checkpoint but also as a mediator of DNA damage repair.

DNA damage response pathways contain many mediator proteins, such as Rad9, Mrc1, Claspin, and 53BP1, which are important for the integration and amplification of DNA damage signals (1). Recent studies suggest that mediator of DNA damage checkpoint protein 1 (MDC1)[1] regulates many aspects of DNA damage response pathways, such as intra-S phase checkpoint, G_2/M checkpoint, and radiation-induced apoptosis (2–8). Many proteins involved in DNA damage response pathways, such as ATM, BRCA1, Chk2, NBS1/MRE11/Rad50, interact with MDC1 (2–6). In response to DNA damage, MDC1 forms nuclear foci at the sites of DNA damage. The formation of MDC1 foci requires H2AX (2, 6), with MDC1 in turn regulating foci formation of NBS1/MRE11/Rad50 and BRCA1 (2, 3, 5). In addition, MDC1 regulates the phosphorylation of BRCA1, Chk1, and SMC1 in response to ionizing radiation (2, 5).

Similar to other mediator proteins, MDC1 contains several protein-protein interaction domains, such as the FHA domain and the BRCT domain. It is believed that MDC1 functions as an adaptor protein, recruiting downstream proteins to upstream kinases, such as ATM/ATR, and facilitating signal transduction following DNA damage. In addition to the FHA domain and BRCT domain, MDC1 contains 14 repeat sequences (aa 1124–1697), with no apparent homology to other known proteins in the data base. We hypothesized that the repeat region of MDC1 is also involved in protein-protein interaction. Here, we report that this repeat region of MDC1 directly interacts with Ku/DNA-PK, and this interaction is required for efficient DNA-PK autophosphorylation and DNA damage repair.

EXPERIMENTAL PROCEDURES

Cells, Constructs, and Antibodies—HeLa cells were purchased from National Cell Culture Center (Minneapolis, MN). V3.3, V3.155, CHO-K1, and XRS6 cells were kindly provided by Dr. Penny Jeggo. GST-MDC1 repeat was generated by subcloning a fragment of MDC1 cDNA encoding aa 1148–1610 into pGex5X3 vector. Wild-type and deletion mutation (ΔR) of MDC1 were cloned in pcDNA3.1V5His vector (Invitrogen). The sequence of MDC1 siRNA was described previously (4). Rabbit anti-MDC1, anti-phospho-Thr2609DNA-PK and anti-γH2AX antibodies were described previously (5, 9). Anti-MDC1 monoclonal antibodies were generated by Mayo Antibody Core Facility using standard procedure. Anti-DNA-PK antibodies were purchased from NeoMarkers (Fremont, CA). Anti-Ku80 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY).

Double Strand Break (DSB) Repair Assay—Cells were twice transfected with control siRNA or MDC1 siRNA as described previously (5). 72 h after initial transfection, cells were irradiated (40 Gy) and left to recover for the indicated time. Cells were then trypsinized and resuspended in agarose plugs. The plugs were loaded onto 0.8% agarose gel, and pulse field gel electrophoresis was performed.

Random Integration Assay—Cells were transfected twice with control siRNA or MDC1 siRNA as described previously (5). At the time of second siRNA transfection, cells were cotransfected with linearized pIRES-Puro, kindly provided by Dr. Christopher Ward (Mayo Clinic). 24 h later, cells were plated at different densities in media containing puromycin (2 μg/ml), and colony formation was determined 7–10 days later.

Microhomology Repair Assay—Cells were transfected twice with control siRNA, DNA-PK siRNA, or MDC1 siRNA as described previously (5). Microhomology assay was performed as described previously (10).

RESULTS AND DISCUSSION

To identify proteins that interact with the repeat region of MDC1, we used a GST-MDC1 fragment containing most of the repeat sequence (aa 1148–1610) as an affinity matrix to purify proteins that may associate with MDC1. We identified several

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

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[1] The abbreviations used are: MDC1, mediator of DNA damage checkpoint protein 1; aa, amino acids; siRNA, small interfering RNA; Gy, gray; DSB, double strand break; IR, irradiation; GST, glutathione *S*-transferase; FHA, forkhead-associated; BRCT, BRCA1 carboxyl-terminal; NHEJ, non-homologous end joining.

proteins, especially a protein larger than 250 kDa that specifically interacts with the GST-MDC1 repeat region (*GST-MDCR*) (Fig. 1*A*). Mass spectrometry and microsequencing revealed this protein as DNA-PKcs, which is the catalytic subunit of DNA-PK. DNA-PKcs and Ku, the regulatory factors that are composed of heterodimer of 70 and 80 kDa, respectively, form catalytically active DNA-PK (11). Western blot confirmed that GST-MDC1 repeats efficiently pulled down DNA-PKcs and Ku from cell lysates (Fig. 1*B*). To prove that DNA-PK and MDC1 interact *in vivo*, we performed coimmunoprecipitation experiment. As shown in Fig. 1*C*, MDC1 coimmunoprecipitated with DNA-PKcs and Ku. No Ku or DNA-PKcs coimmunoprecipitated with normal rabbit serum (*NRS*) (Fig. 1*C*) or with anti-53BP1, anti-BRCA1, and anti-Claspin antibodies (data not shown), suggesting the specificity of MDC1-DNA-PKcs/Ku interaction. The interaction of MDC1 and DNA-PKcs/Ku complex is not dependent upon DNA, since ethidium bromide (100 μg/ml) does not affect the coimmunoprecipitation of MDC1 and DNA-PKcs/Ku (data not shown). Furthermore, the interaction of MDC1 and DNA-PKcs/Ku appears to be constitutive, since we did not observe any changes in this interaction before or after DNA damage (data not shown). We next asked whether the repeat region of MDC1 is required for MDC1-Ku interaction *in vivo*. Constructs encoding V5 tagged wild-type MDC1 or MDC1 containing a deletion of the repeat region (MDC1delR) were expressed in 293T cells. As shown in Fig. 1*D*, the binding of Ku to MDC1delR is significantly decreased when compared with its binding to wild-type MDC1, suggesting that the repeat region of MDC1 is important for its interaction with Ku/DNA-PK *in vivo*.

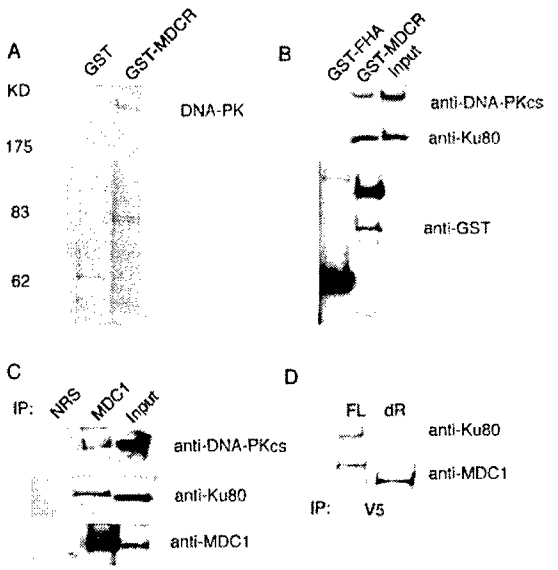

FIG. 1. **MDC1 associates with DNA-PKcs/Ku complex through the repeat region of MDC1.** *A*, Coommassie staining of proteins associated with GST-MDC1 repeat region (*GST-MDCR*) or GST control affinity column. *B*, GST-MDCR was incubated with HeLa cell lysate, and proteins bound to GST-MDCR were separated by SDS-PAGE and immunoblotted with anti-DNA-PKcs or anti-Ku80 antibodies. *C*, HeLa cells were lysed, and cell lysates were immunoprecipitated (*IP*) with normal rabbit serum (*NRS*) or anti-MDC1 antibodies and blotted with indicated antibodies. Inputs represent 7% of total proteins used in immunoprecipitation. *D*, HeLa cells were transfected with plasmids encoding V5-tagged wild-type MDC1 or MDC1 with a deletion of the repeat region (*dR*). 48 h later, cells were collected and cell lysates were immunoprecipitated (*IP*) with anti-V5 antibodies, followed by immunoblotting with anti-Ku80 or anti-MDC1 antibodies.

Because Ku specifically interacts with DNA ends, it is suggested that DNA-PKcs is activated and recruited to DSB through its interaction with the Ku heterodimer (12, 13). However, the mechanism of DNA-PK activation is not clear. Recent studies suggest that DNA-PKcs undergoes autophosphorylation at multiple sites (9, 14–16), and the autophosphorylation of DNA-PKcs is important to DSB repair and cell survival following IR (9, 15, 16). Furthermore, phospho-DNA-PKcs foci colocalize with γH2AX and 53BP1 foci after DNA damage (9), suggesting that phosphorylated DNA-PK localizes at the sites of DNA breaks. As expected, MDC1 foci colocalize with phospho-Thr2609-DNA-PKcs (pT2609DNA-PK) foci following IR (Fig. 2*A*). No pT2609DNA-PK foci were detected in DNA-PK-deficient cell line (Ref. 9 and supplementary Fig. S1), suggesting the specificity of the anti-pT2609DNA-PK antibodies. Several lines of evidence suggest that MDC1 foci formation is independent of DNA-PKcs/Ku. A previous study has shown that DNA-PKcs is not required for MDC1 foci formation following IR (3). We also observed normal MDC1 and γH2AX foci formation in DNA-PK-deficient cells (data not shown). In addition, we observed normal MDC1 foci formation in Ku-deficient Xrs6 cells (data not shown). Interestingly, down-regulation of MDC1 expression using siRNA significantly decreased pT2609DNA-PK foci formation following IR (Fig. 2*B*). The decreased phospho-DNA-PK foci in MDC1-depleted cells could be

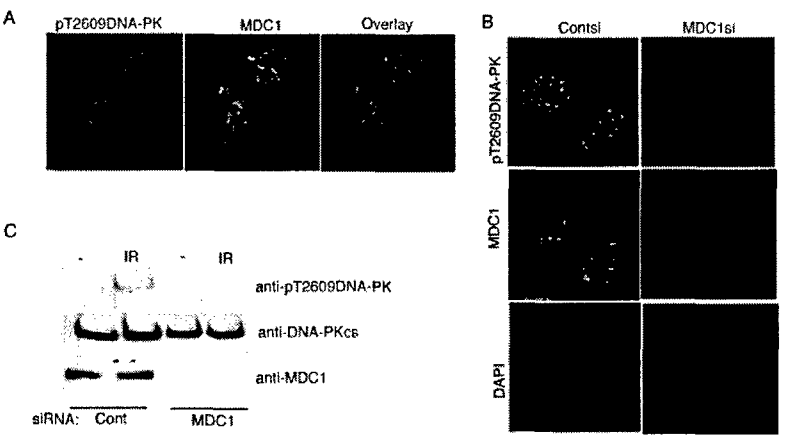

FIG. 2. **MDC1 regulates DNA-PKcs phosphorylation following DNA damage.** *A*, MDC1 co-localizes with phospho-DNA-PKcs following IR. Irradiated HeLa cells (3 Gy) were fixed 1 h later and immunostained with anti-MDC1 or anti-pT2609DNA-PK antibodies. *B*, HeLa cells were transfected twice with control siRNA (*Contsi*) or MDC1 siRNA. 72 h after initial transfection, cells were irradiated (3 Gy) and collected 1 h later. Cells were fixed and immunostained with anti-MDC1 or anti-p2609DNA-PK antibodies. *C*, HeLa cells were transfected twice with control (*Cont*) or MDC1 siRNA. 72 h later, cells were irradiated (5 Gy) or left untreated. 30 min later, cells were collected, and DNA-PK was immunoprecipitated and blotted with anti-p2609DNA-PK or anti-DNA-PK antibodies (*upper two panels*). Alternatively, cell lysates were blotted with anti-MDC1 to demonstrate the down-regulation of MDC1 (*lower panel*).

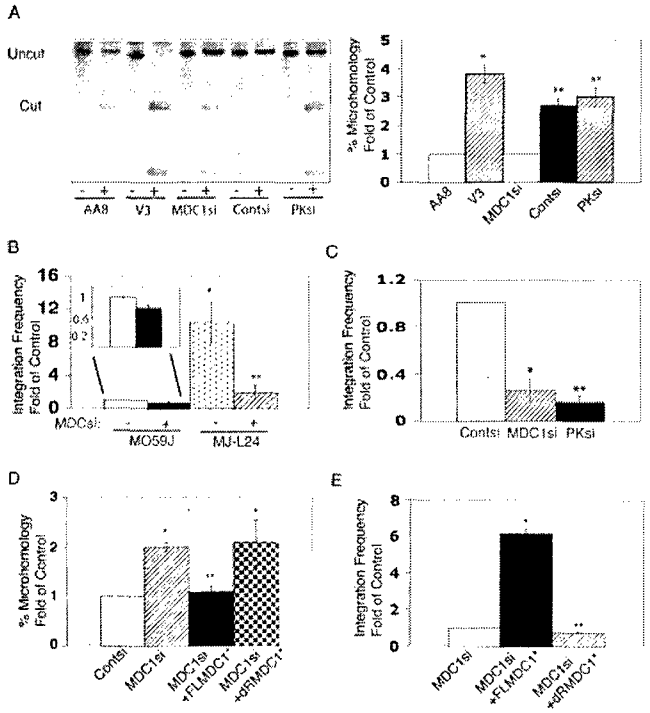

FIG. 3. **MDC1 is involved in DNA-PK-dependent NHEJ repair.** *A*, direct and microhomology-mediated end-joining assays were performed. HeLa cells were transfected with the indicated siRNA together with pDVG94 digested with EcoR47III and EcoRV. DNA-PK proficient AA8 and DNA-PK-deficient cells V3 were included as a control. Plasmid DNA was then extracted and used as template for PCR reaction with primers surrounding the microhomology region. Equal amounts of PCR products were digested with BstXI, and the products were separated on a polyacrylamide gel. The density of digested (microhomology-based joining) and undigested (direct joining) fragments were quantified, and the percentage of microhomology-mediated joining was obtained. The results were expressed as fold increase of microhomology-mediated joining compared with that of control sample. Statistical analysis was performed. *, $p < 0.02$; **, $p < 0.05$. *B* and *C*, random plasmid integration assay was performed. M059J cells (DNA-PK-deficient), MJ-L24 (DNA-PK-proficient), or HeLa cells were transfected with indicated siRNA and linearized pIRES-Puro. Cells were then grown in selection media containing puromycin for 10 days, and colony numbers were determined. The results were expressed as percentages of control, with control siRNA transfected sample as 100%. Statistical analysis was performed. *, $p < 0.001$; **, $p < 0.001$. *D* and *E*, A459 cells (*D*) or HeLa cells (*E*) transfected with MDC1 siRNA were infected with lentiviruses encoding siRNA-resistant full-length MDC1 (MDC1*) or MDC1 with a deletion of the repeat region (MDC1dR*). The cells were then used to assay microhomology-mediated end-joining (*D*) or plasmid integration (*E*). Statistical analysis was performed. *, $p < 0.05$; **, $p > 0.1$. *MDC1si*, MDC1 siRNA; *Contsi*, control siRNA; *PKsi*, DNA-PK siRNA.

caused by defective recruitment of phospho-DNA-PK at the sites of DNA damage or decreased DNA-PK phosphorylation. Therefore, we next examined whether DNA-PKcs phosphorylation requires MDC1. As shown in Fig. 2*C*, DNA-PKcs phosphorylation at Thr^{2609}, but not DNA-PKcs expression, is decreased in cells transfected with MDC1 siRNA. We also detected decreased DNA-PK phosphorylation at Ser^{2056} site, another damage-induced phosphorylation site on DNA-PK (data not shown). However, DNA-PK kinase activity was not affected by the depletion of MDC1 (supplementary Fig. S2). Taken together, these results suggest that MDC1 regulates DNA-PKcs autophosphorylation following IR.

Many genetic and biochemical studies have established an essential role of DNA-PK in DSB repair (17). The autophosphorylation of DNA-PKcs is important for DSB repair and cell survival in response to ionizing radiation (9, 15, 16). Since MDC1 regulates DNA-PKcs autophosphorylation, it is possible that MDC1 regulates DNA-PK-dependent DSB repair. We performed pulse field gel electrophoresis to assay the kinetics of DSB repair in cells transfected with control siRNA or MDC1 siRNA. We observed a mild defect in DSB repair in MDC1 siRNA-transfected cells (supplementary Fig. S3), suggesting that MDC1 is not essential for overall DSB joining but is instead involved in certain aspects of DNA end-joining. To investigate whether MDC1 is involved in error-free end-joining mediated by DNA-PK, we used a previously established plasmid-based end-joining assay for non-homologous end joining (NHEJ) (10). In this assay, linearized pDVG94 plasmid was used to transfect cells. If cells are defective in direct error-free end-joining, an alternative microhomology-based joining will be used, therefore creating a BstXI site. In cells that contain mutations of DNA-PK, Ku, XRCC4, or DNA ligase IV, a dramatic increase in the alternative microhomology-based joining has been observed (10), suggesting the specific requirement of Ku/DNA-PK in an error-free end-joining repair pathway. As shown in Fig. 3*A*, in MDC1 siRNA transfected cells, significantly more end-joining occurred using the alternative pathway, similar to the cells transfected with DNA-PK siRNA or that in DNA-$PK^{-/-}$ cells (V3) (Fig. 3*A*). These results suggest that MDC1 is involved in DNA-PKcs/Ku dependent error-free end-joining. The repair defect observed in cells transfected with MDC1 siRNA is not due to any change in cell cycle progression, since we do not observed any detectable difference in cell cycle profile with or without MDC1 siRNA treatment (data not shown). To further confirm our observations, we next used random plasmid integration assay, another assay for DNA-PK-dependent end-joining activity (18, 19). Linearized DNA with selection marker is used to transfect cells, and the efficiency of random chromosomal integration of the plasmid DNA is measured by colony formation in selection media. Consistent with previous findings (18, 19), the integration frequency of the puromycin-resistant gene is significantly lower in DNA-PK-deficient cells than that observed in the wild-type parental cell line (Fig. 3*B*). Knock-down MDC1 using siRNA resulted in a significant decrease of integration frequency in DNA-PK-proficient cells but only a minor reduction in DNA-PK-deficient cells (Fig. 3*B*). The integration frequency was also significantly lower in HeLa cells transfected with MDC1 siRNA or DNA-PKcs siRNA, compared with that in cells transfected with control siRNA (Fig. 3*C*). Taken together, these results suggest a role of MDC1 in DNA-PK-dependent DSB repair.

Since MDC1 has multiple binding partners, depletion of MDC1 might have an indirect effect on DSB repair. To demonstrate that the interaction between DNA-PKcs/Ku and MDC1 is important for DNA-PKcs phosphorylation and DNA end-joining, we generated constructs encoding siRNA-resistant full-length MDC1 (MDC1*) and MDC1 with deleted repeat region (MDC1delR*). Reconstituting MDC1 knock-down cells with full-length (MDC1*), but not deletion mutation of MDC1 (MDC1delR*), restored pT2609DNA-PK foci formation, suggesting that the interaction of MDC1 and DNA-PKcs/Ku is important for DNA-PK phosphorylation (supplementary Fig. S4). Consistent with DNA-PK phosphorylation, reconstitution of full-length MDC1 re-established normal DSB joining (Fig. 3*D*) and plasmid integration (Fig. 3*E*), while reconstitution of MDC1delR* failed to do so. As a control, we evaluated NBS1 foci formation in cells reconstituted with MDC1* or MDC1delR*. In agreement with an early study suggesting that NBS1 interacts with MDC1 FHA domain (3), reconstitution of either full-length MDC1* or MDC1delR* restored NBS1 foci formation in response to IR (supplementary Fig. S5). Furthermore, reconstitution of either full-length MDC1* or MDC1delR* restored the intra-S checkpoint in response to IR (data not shown), suggesting that MDC1delR* has intact

checkpoint function. These data support that the specific interaction between MDC1 and DNA-PKcs/Ku plays an important role in DNA end-joining.

We report here that in addition to the FHA and BRCT domain, the repeat region of MDC1 is involved in protein-protein interactions. It directly binds Ku and regulates DNA-PKcs autophosphorylation. Previously, DNA-PKcs autophosphorylation has been shown to be Ku-dependent, presumably facilitated by oligomerization of DNA-PKcs/Ku at DNA ends (9). Currently, we could not evaluate the effect of MDC1 on the recruitment of DNA-PKcs/Ku to DNA ends, since we have not been able to detect DNA-PKcs or Ku foci formation in response to IR nor could we detect increased binding of DNA-Pkcs/Ku on chromatin (data not shown). Since Ku binds DNA ends with very high affinity, we speculate that MDC1 may not be required for initial binding of Ku and DNA-PKcs to DNA ends. Instead, MDC1 might help the oligomerization and stabilization of DNA-PKcs/Ku complexes at the sites of DNA damage. A similar role for MDC1 has been suggested for the sustained binding of NBS1/MRE11/Rad50 to DNA ends (20). An emerging theme in DNA damage signaling is that mediators such as NBS1 and MDC1 are important for ATM phosphorylation and activation by facilitating the concentration of ATM at the sites of DNA damage (21–23). We propose that MDC1 may play a similar role in regulating the autophosphorylation of DNA-PKcs following DNA damage.

DNA-PKcs phosphorylation is important for DSB repair (9, 15, 16). Mutation of DNA-PKcs autophosphorylation sites affects cell survival following radiation and also error-free end-joining. However, DNA-PKcs with mutations at these phosphorylation sites could still support DNA end-joining using an alternative pathway that leads to mutations and deletions (15, 16). Consistent with this notion, cells depleted of MDC1 show a mild defect in overall DSB joining when analyzed by pulse field gel electrophoresis, suggesting that overall DSB joining activity does not require MDC1. Interestingly, cells depleted of MDC1 show significant decrease in the frequency of plasmid integration into chromosomes (Fig. 3*C*), which has been shown to be dependent on DNA-PK (18, 19). In addition, using a plasmid-based assay, we show that cells depleted of MDC1 often use alternation error-prone pathway to repair DSBs (Fig. 3*A*), suggesting that MDC1 is involved in a DNA-PK-mediated error-free end-joining pathway.

In summary, we have shown for the first time that the repeat region of MDC1 is a protein-protein interaction domain and binds directly to Ku/DNA-PK. We also demonstrate that MDC1 is critical for the efficient IR-induced autophosphorylation of DNA-PK, which in turn facilitates DSB repair. These data suggest that in addition to be a mediator of DNA damage checkpoint, MDC1 also contributes to DNA damage repair.

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REFERENCES

1. Sancar, A., Lindsey-Boltz, L. A., Unsal-Kaccmaz, K., and Linn, S. (2004) *Annu. Rev. Biochem.* **73,** 39–85
2. Stewart, G. S., Wang, B., Bignell, C. R., Taylor, A. M. R., and Elledge, S. J. (2003) *Nature* **421,** 961–966
3. Goldberg, M., Stucki, M., Falck, J., D'Amours, D., Rahman, D., Pappin, D., Bartek, J., and Jackson, S. P. (2003) *Nature* **421,** 952–956
4. Lou, Z., Minter-Dykhouse, K., Wu, X., and Chen, J. (2003) *Nature* **421,** 957–961
5. Lou, Z. K., Chini, C. C. S., Minter-Dykhouse, K., and Chen, J. J. (2003) *J. Biol. Chem.* **278,** 13599–13602
6. Peng, A., and Chen, P. L. (2003) *J. Biol. Chem.* **278,** 8873–8876
7. Xu, X., and Stern, D. F. (2002) *J. Biol. Chem.* **278,** 8795–8803
8. Shang, Y., Bodero, A., and Chen, P. (2002) *J. Biol. Chem.* **278,** 6323–6329
9. Chan, D. W., Chen, B. P. C., Prithivirajsingh, S., Kurimasa, A., Story, M. D., Qin, J., and Chen, D. J. (2002) *Genes Dev.* **16,** 2333–2338
10. Verkaik, N. S., Esveldt-van Lange, R. E., van Heemst, D., Bruggenwirth, H. T., Hoeijmakers, J. H., Zdzienicka, M. Z., and van Gent, D. C. (2002) *Eur. J. Immunol.* **32,** 701–709
11. Smith, G. C., and Jackson, S. P. (1999) *Genes Dev.* **13,** 916–934
12. Gottlieb, T. M., and Jackson, S. P. (1993) *Cell* **72,** 131–142
13. Dvir, A., Peterson, S. R., Knuth, M. W., Lu, H., and Dynan, W. S. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 11920–11924
14. Douglas, P., Sapkota, G. P., Morrice, N., Yu, Y. P., Goodarzi, A. A., Merkle, D., Meek, K., Alessi, D. R., and Lees-Miller, S. P. (2002) *Biochem. J.* **368,** 243–251
15. Ding, Q., Reddy, Y. V., Wang, W., Woods, T., Douglas, P., Ramsden, D. A., Lees-Miller, S. P., and Meek, K. (2003) *Mol. Cell. Biol.* **23,** 5836–5848
16. Soubeyrand, S., Pope, L., Pakuts, B., and Hache, R. J. G. (2003) *Cancer Res.* **63,** 1198–1201
17. Jackson, S. P. (1997) *Int. J. Biochem. Cell Biol.* **29,** 935–938
18. Smith-Ravin, J., and Jeggo, P. A. (1989) *Int. J. Radiat. Biol.* **56,** 951–961
19. Harrington, J., Hsieh, C. L., Gerton, J., Bosma, G., and Lieber, M. R. (1992) *Mol. Cell. Biol.* **12,** 4758–4768
20. Lukas, C., Melander, F., Stucki, M., Falck, J., Bekker-Jensen, S., Goldberg, M., Lerenthal, Y., Jackson, S. P., Bartek, J., and Lukas, J. (2004) *EMBO J.* **23,** 2674–2683
21. Uziel, T., Lerenthal, Y., Moyal, L., Andegeko, Y., Mittelman, L., and Shiloh, Y. (2003) *EMBO J.* **22,** 5612–5621
22. Carson, C. T., Schwartz, R. A., Stracker, T. H., Lilley, C. E., Lee, D. V., and Weitzman, M. D. (2003) *EMBO J.* **22,** 6610–6620
23. Mochan, T. A., Venere, M., DiTullio, R. A., Jr., and Halazonetis, T. D. (2003) *Cancer Res.* **63,** 8586–8591